Antibody Responses to the Hepatitis C Virus E2 Protein: Relationship to Viraemia and Prevalence in Anti-HCV Seronegative Subjects

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A small proportion of patients with chronic hepatitis C virus (HCV) infection show no serological responses to the HCV polypeptides incorporated in commercial III generation immunoassays. To determine whether sera from these subjects contain antibodies to the highly immunoreactive second envelope polypeptide E2, which is not included in current anti-HCV assays, we studied 59 anti-HCV negative subjects who were found consistently to be HCV RNA positive by polymerase chain reaction (PCR). Controls included 167 anti-HCV seropositive patients with or without serum HCV RNA and normal subjects. Antibodies to the E2 region were sought for by ELISA using the following antigens: a full length E2 protein expressed in insect cells using a baculovirus vector and extracted under denaturing conditions (dE2), and a C-terminal truncated soluble E2 (sE2) protein (a.a. 390–683), also expressed with a baculovirus vector, containing a signal peptide of rabies virus G protein which allows its secretion into the culture supernatant. Sera from only two (3.4%) of the 59 anti-HCV negative, HCV RNA positive patients recognised sE2 and none dE2. In sharp contrast, 82% of seropositive, viraemic patients recognised sE2 and 60% dE2, the difference in immunoreactivity being statistically significant ($P < 0.0003$). A significantly lower proportion of sera from anti-HCV positive, HCV RNA negative subjects recognised either sE2 or dE2 (16% and 13%, respectively, $P < 0.000001$). Healthy controls were consistently negative. These results indicate that antibody responses to predominantly conformational epitopes on the HCV E2 protein are common in patients with chronic HCV infection and are strictly related to the presence of circulating viral genomes. In contrast, only a minor proportion of HCV RNA positive patients, but anti-HCV seronegative by commercial immunoassays, have humoral immune responses to the HCV E2 region. ***J Med Virol 51: 1–5, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Hepatitis C virus (HCV) is a highly heterogeneous RNA virus which causes acute and chronic liver disease and hepatocellular carcinoma [Alter, 1995]. Diagnosis of HCV infection can be established readily by sensitive and specific serological assays which incorporate a mixture of viral polypeptides on the solid phase. These antigens include both structural proteins, such as the putative nucleocapsid protein, and nonstructural region polypeptides, such as NS3, NS4, and NS5. HCV envelope proteins have never been included in commercial immunoassays, presumably because of the considerable sequence heterogeneity among viral isolates, which would theoretically preclude identification of anti-envelope antibodies in patients infected with viral variants having significant amino acid sequence divergence from the prototype viral sequence utilized as antigen. Evi-

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dence in support of this hypothesis comes from the report of antibody responses restricted to specific viral isolates [Spaete et al., 1992; Weiner et al., 1992; Kato et al., 1993; Farci et al., 1995]. On the other hand, humoral immune responses against HCV envelope proteins are often detected in patients with chronic HCV infection with circulating HCV RNA [Inoue et al., 1992; Mita et al., 1992; Chien et al., 1993; Hsu et al., 1993; Scarselli et al., 1995]. Recently, we provided evidence for the existence of cross-reactive antibodies to the hypervariable region 1 (HVR1) of HCV in a significant proportion of patients with documented chronic HCV infection, but in none of the non-viremic anti-HCV positive subjects [Scarselli et al., 1995]. The broad reactivity of HCV envelope glycoproteins with sera from patients with chronic HCV infection suggests that it may be possible to detect anti-envelope antibodies in some viremic patients who apparently lack circulating anti-HCV antibodies by commercial immunoassays. To this end, we examined the prevalence of antibodies to the highly immunoreactive E2 polypeptide in a cohort of patients without serological evidence of exposure to HCV by commercial immunoassays but who were nonetheless consistently positive for serum HCV RNA by the polymerase chain reaction (PCR). Anti-HCV seropositive subjects with and without circulating HCV RNA served as controls.

MATERIALS AND METHODS

Patients

Two-hundred and twenty six patients were included in this study. Patients were drawn from a large database of 3,256 subjects tested for the presence of serum HCV RNA, of whom 436 (13%) were anti-HCV seronegative. In the latter, HCV RNA was sought for because of unexplained, persistently raised alanine aminotransferase (ALT) levels or because they belonged to categories at high risk of acquiring HCV infection. Fifty-nine subjects (37 males, median age 16 years, age range 4 months–70 years) who were repeatedly anti-HCV negative by III generation ELISA (Ortho Diagnostic System, Raritan, NJ) but HCV-RNA positive by nested reverse transcription polymerase chain reaction (RT PCR) using conserved primers from the 5′ non-coding (NC) region [Silini et al., 1993], were selected from this group. At least two PCR determinations were carried-out over a 6-month period with concordant results. Fifteen patients had been in remission from acute lymphoblastic leukaemia (ALL) for a median of 24 months, range 2 to 153 months after treatment withdrawal and 9, also with ALL, were treated with cytotoxic drugs at the time of the study. Seven of these had elevated ALT. Of the remainder, 15 patients had persistently elevated ALT and 20 were asymptomatic subjects with persistently normal or near normal ALT levels (fluctuations within 5 U/litre above the upper limit of normal of 40 U/litre) who underwent a RT-PCR test for the following reasons: one was a possible kidney donor, two were organ transplant recipients, three were pharmacologically immunosuppressed, two were on haemodialysis, four were born from anti-HCV positive mothers, and eight were blood donors who were referred to our centre because they showed border line values by ELISA. With the exception of the 24 leukaemic patients who received blood transfusions, it was possible to identify risk factors of acquiring HCV infection in 12 patients only: three patients received blood transfusions, four were vertically infected, one had sexual contacts, one was an HIV-positive intravenous drug abuser, and three patients were recipients of a heart transplant and were pharmacologically immunosuppressed. Seven of these 59 patients underwent diagnostic liver biopsy, which showed mild chronic active hepatitis (CAH) in one patient, chronic persistent hepatitis (CPH) in one patient, cirrhosis in one patient, fatty liver in one patient, and normal liver in three patients.

The second group consisted of 167 (100 males, median age 50 years, age range 8–73 years) consecutive anti-HCV positive patients, of whom 122 were also serum HCV RNA positive. Elevated ALT were present in 68 of the 122 anti-HCV positive, PCR positive patients. Risk factors of acquiring HCV infection were identified in 27 subjects only: eight received blood transfusions, 12 were former intravenous drug abusers (IVDA), five were exposed to sexual or intrafamiliar contacts, and two were health care workers. Fifty-two patients from this group underwent diagnostic liver biopsy which showed CAH in 33 patients, CPH in 10, cirrhosis in two, minimal histological changes in one, hepatocellular carcinoma (HCC) in one, and normal liver in five patients. With respect to the 45 anti-HCV positive subjects without serum HCV RNA, risk factors were identified in eight subjects only: three were health care workers, two had a heart transplant, one received blood transfusions, one was IVDA, and one had sexual contacts with an anti-HCV positive partner. A liver biopsy was performed in five patients and showed CAH in three, CPH in one and normal liver in one.

To avoid potential contamination by carry-over of PCR products and false-positive results, a strict separation was observed for pre- and post-PCR procedures which were carried out in different laboratories. Aliquotted reagents, aerosol-free tips, and all other recommended precautions were also used [Kwok and Higuchi, 1989]. An adequate number of controls was also included in each set of experiments.

HCV Typing

HCV genotyping was undertaken by PCR amplification of core region sequences with universal and type-specific primers to generate DNA fragments of different sizes, specific for the five most common HCV genotypes [Silini et al., 1995; Silini and Mondelli, 1995]. The genotype 2 a/c-specific primer was modified because of several nucleotide differences between Italian and Japanese isolates of the genotype 2 a/c sequence.

Antigens

Two different preparations of E2/NS1 protein were used as antigens: a full length E2 protein expressed in

Spodoptera frugiperda (Sf9) cells using a recombinant baculovirus and extracted under denaturing condition (dE2) and a C-terminal truncated soluble E2 (sE2) protein (aa 390–683), also expressed in insect cells infected with recombinant baculovirus, containing a signal peptide of rabies virus G protein which allows its secretion into the culture supernatant (Nishihara et al., 1993). The supernatant of insect cells infected with wild-type baculovirus (baculo wt) served as control.

Purification of Denatured E2 (dE2)

A cDNA fragment from a HCV type 1b isolate encoding the full-length glycosylated envelope protein E2 was cloned into the baculovirus transfer vector pAcAS3, allowing expression of the protein in Sf9 cells under the control of the p10 promoter as described elsewhere [Vlak et al., 1990]. The recombinant E2 protein was purified to over 80% under denaturing conditions which consisted in multiple extractions with 0.1M sodium carbonate buffer pH 11.5 containing 50 mM dithiothreitol (DTT) and final solubilization in 50 mM Tris buffer pH 8.0 with 10 g/L SDS and 1M Urea. This preparation was further purified by SDS-polyacrylamide gel preparative electrophoresis [Goding, 1986]. The purified protein preparation was characterized by SDS-PAGE and Western blot. Fractions containing high purity E2 protein were pooled and dialysed against Tris buffer for use in ELISA.

Purification of C-Terminal Truncated Soluble E2 (sE2)

The recombinant sE2 protein also derived from a HCV genotype 1b isolate (GenBank/EMBL/DDBJ accession No. D13406) was purified by metal chelating affinity chromatography from High-V cells infected with the recombinant baculovirus VLKGss3031 [Nishihara et al., 1993]. Briefly, 250 ml of Sf 900 II (Gibco, Grand Island, NY) medium from infected cells were concentrated 10 times by ultrafiltration on Centriprep 10 (Amicon, Beverly, MA), mixed with an equal volume of buffer A (10 mM N-2-hydroxyethylpiperazine-N′-2 ethane sulphonic acid (HEPES) pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 830 mM NaCl, 1 mM dithiothreitol (DTT), 17% glycerol, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM NaF), containing 1.6 mM imidazole, and incubated for 45 min at 4°C. The sample was then centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was loaded onto a 1 ml Hitrap Chelating column (Pharmacia, LKB Biotechnology, Uppsala, Sweden) equilibrated previously in buffer A with 1.6 mM imidazole. The column was sequentially washed with 3 ml of buffer D (10 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1 mM DTT, 17% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF) containing 0.8 mM imidazole, 3 ml of buffer D containing 8 mM imidazole and 3 ml of buffer D containing 30 mM imidazole. The protein was then eluted with 3 ml of buffer D containing 80 mM imidazole, dialysed against PBS and stored at −80°C.

ELISA

Sera were diluted 1:100 in blocking buffer containing 5% of 1:1 (vol./vol.) solution of baculo wt supernatant and MOC transfection medium and preincubated for 2 hours at room temperature, to avoid possible cross-reactions with insect proteins. Elisa plates (96 wells, LINBRO, ICN Biomedicals, Horsham, PA) were coated overnight at 4°C, with: i, 1.25 μg/ml of sE2; ii, 2 μg/ml of dE2, and iii, Baculo wt supernatant 2% in 50 mM $NaHCO_3$ pH 9.6. Plates were then washed four times with 0.05% Tween 20/PBS and incubated for 1 hour at 37°C with 200 μl/well of 0.5% non-fat dry milk, 0.1% Tween-20 in PBS (blocking buffer) to prevent non-specific binding. 100 μl of blocked sera were distributed in the plates and incubated for 2 hours at room temperature. After four washings with 0.05% Tween-20/PBS, bound IgG were detected by a peroxidase conjugate (DAKO P214, Dako, Copenhagen, DK) after incubation for 1 hour at 37°C. The reaction was developed with *ortho*-phenylendiamine-HCl as substrate and A_{492} determined after a further washing with 0.05% Tween 20/PBS. Sera from 10 healthy subjects served as negative controls for each microtitre plate. The mean A_{492} values for negative controls were as follows: sE2, 0.111 ± 0.047 and dE2, 0.129 ± 0.046. The upper limit of normal in such a system, five standard deviations above the mean value for negative controls for each antigen, was 0.347 for sE2 and 0.359 for dE2.

RESULTS

Sera from only two (3.4%) of the 59 anti-HCV negative, HCV RNA positive subjects recognised sE2, and none recognised dE2 (Table I). In contrast, sera from 100 (82%) of 122 HCV seropositive, viremic patients recognised sE2 and 73 (60%) recognised dE2, the difference being statistically significant ($\chi^2 = 13.4$, $P < 0.0003$). Sera from 68 of the 73 subjects recognising dE2 were also reactive with sE2. A significantly lower proportion of sera from the 45 anti-HCV positive, HCV RNA negative patients showed reactivity for sE2 and dE2: 7 (16%) and 6 (13%), $P = 0$ and $P < 0.0000002$ respectively, compared to anti-HCV positive, HCV RNA positive subjects (Table I).

When the antibody response to HCV envelope proteins was analysed according to the infecting HCV genotype in the 104 anti-HCV positive patients in whom this information was available, the highest proportion of sera reactive with sE2 was found in patients infected with type 1b (Table II). The difference in the proportion of immunoreactive sera with sE2 reached statistical significance only when patients infected with type 1b were compared with those infected with type 3a ($\chi^2 = 6.7$, $P < 0.01$). In addition, the overall proportion of sera reactive with dE2 was again significantly lower than that of sera reactive with sE2 ($\chi^2 = 15$; $P < 0.0002$) (Table II). The single patient infected with type 2b was not considered for statistical analysis.

HCV genotypes were determined in 12 of the 59 anti-HCV negative patients: six were infected by HCV type

TABLE I. Anti-HCV Envelope Antibodies in Patients Exposed to HCV[a]

Anti-HCV	HCV-RNA	sE2	dE2
Positive (n. = 167)	positive	100/122 (82%)[b]	73/122 (60%)[c]
	negative	7/45 (16%)	6/45 (13%)
Negative (n. = 59)	positive	2/59 (3.4%)	0/59 (0%)

[a]sE2: C-terminal truncated soluble E2. dE2: Full-length denatured E2. HCV RNA positive vs. HCV RNA negative in patients anti-HCV postive: [b]$P = 0$; [c]$P < 0.0000002$.

TABLE II. Anti-Envelope Antibodies in 104 Anti-HCV Positive, HCV RNA Positive Patients With Known HCV Genotype[a]

GENOTYPE	sE2	dE2
1b	32/34 (94%)	19/34 (56%)
2 a/c	39/44 (89%)	28/44 (64%)
2b	1/1 (100%)	0/1 (0%)
3a	16/25 (64%)	14/25 (56%)

[a]sE2: C-terminal truncated soluble E2. dE2: Full-length denatured E2. Reactivity to sE2 vs. dE2: χ^2 15; $P < 0.0001076$.

1b, four by type 2 a/c, one by type 1a, and one had a mixed infection with types 1a and 1b. The antibody response to HCV envelope proteins was not analysed according to HCV genotype in this group as only two patients recognised sE2 and the infecting genotype was known in one subject only (type 1b).

None of the sera examined in this study showed antibodies against wild-type baculovirus proteins.

DISCUSSION

Humoral immune responses to Flavivirus envelope glycoproteins have been shown to induce protective immunity in vaccinated hosts [Zhang et al., 1988; Rumenapf et al., 1991]. However, an important limitation to the development of HCV vaccines is that neutralising antibodies are usually isolate-specific [Farci et al., 1992; Farci et al., 1994; Shimizu et al., 1994; Farci et al., 1995]. The region thought to contain the major neutralisation domain(s) of HCV is likely to be located within HVR1, a sequence subjected to an intense immune pressure, the heterogeneity of which may represent a strategy for the virus to escape immune surveillance [Mondelli, 1996]. Recent evidence, however, would suggest that additional neutralising epitopes might be present on the E2 glycoprotein which are distinct from those located in HVR1 [Rosa et al., 1996]. These antibodies are detected by competition of recombinant E2 with sera from HCV-infected patients for binding to a putative cellular receptor. High neutralisation titres are produced following immunisation of primates with recombinant E2, even though low titres of neutralising antibodies are also produced in chronically infected patients. To this end, it is important to emphasize that humoral responses to E2/gp70 have been consistently found in patients with chronic HCV infection [Inoue et al., 1992; Mita et al., 1992; Chien et al., 1993; Hsu et al., 1993; Scarselli et al., 1995; Nisihara et al., 1993]. Why HCV cannot be efficiently neutralised by the host immune response to the E2 protein is still a matter of debate, but it is likely that the extraordinary capability of HCV to mutate rapidly in certain domains is indeed responsible for this phenomenon.

In this study, we have confirmed and extended previous reports showing significant immunoreactivity of the E2/gp70 glycoprotein in a high proportion of patients with chronic HCV infection. More marked immunoreactivity was observed using a C-terminal truncated soluble form of E2, whereas recognition of a full length E2 polypeptide extracted under denaturing conditions resulted in diminished immunoreactivity. Differences in immunoreactivity related to viral genotype cannot be advocated as a possible cause of this phenomenon, since both E2 preparations derived from constructs belonging to the same viral subtype 1b. These findings suggest, instead, that a correct, native conformation of the E2 protein is critical to detect anti-E2 antibodies directed at conformational or discontinuous epitopes [Chien et al., 1993; Ahmed et al., 1996]. Genotype differences, however, may have been responsible for the significantly lower recognition rate of sE2 by sera from patients infected by type 3a compared with those infected by types 1b or 2a/c. Additional important information comes from analysis of antibody responses to E2 according to viraemia. Indeed, the proportion of sera from viraemic anti-HCV carriers recognising E2 preparations was significantly higher than that of sera from non-viraemic anti-HCV carriers. These findings are entirely compatible with preliminary observations by other investigators [Inoue et al., 1992; Mita et al., 1992; Chien et al., 1993; Hsu et al., 1993] and with findings we obtained previously using synthetic peptides derived from HVR1 as antigen [Scarselli et al., 1995]. In that study, recognition of HVR1 sequences was restricted to viraemic subjects, and fine specificity experiments indicated that most sera contained cross-reactive antibodies to a relatively conserved epitope on the HVR1 carboxyl terminal domain [Scarselli et al., 1995]. It is likely that additional epitopes outside HVR1 may also constitute the target of anti-E2 antibodies.

A small porportion of immunocompetent patients with chronic HCV infection shows negative serological responses to the HCV polypeptides incorporated in commercial III generation assays. We have examined whether sera from such patients contained antibodies to the E2 glycoprotein which is not included in current anti-HCV assays. However, only a minor proportion of anti-HCV seronegative, HCV RNA positive patients

showed a humoral immune response to E2 suggesting that inclusion of E2 in the antigenic mixture utilized in HCV screening assay would not significantly improve the sensitivity of serological assays. The reason for such a failure to mount an immune response to the various HCV polypeptides are not immediately apparent. One possible explanation would be that the patients were immunosuppressed at the time of the study, although this may be responsible for immunological non responsiveness in only 24% of the patients. Alternatively, some of the patients with "community-acquired" HCV infection may have been infected at birth at a time when the immune system is thought to be immature. However, it would be impossible to confirm this hypothesis, since the overwhelming majority of acute HCV infections is completely asymptomatic; moreover, immunological tolerance is usually focused towards a specific protein, and it is highly unlikely that it would encompass virtually all HCV proteins. Finally, a further possible explanation would be that another member of the *Flaviviridae* family sharing extensive sequence homology with the 5′-NC region of HCV may have been responsible for the consistently positive PCR signal observed in these subjects. Further studies are currently under way to address this issue.

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